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Abstract

A colorimetric procedure has been developed for assay of the proteolytic activity of bate in drum liquor using hide powder azure, a commercially available substrate. Stock bate powders or solutions can be assayed with the same procedure. Results of the application of the assay to tannery samples are presented and the potential use of the procedure for quality control is discussed. The cost of chemicals and single-use equipment for a single assay is $4.00, and the run time 30 minutes. Fifteen samples can be run by one operator "simultaneously", i.e., with a one minute delay between one sample and the next. The procedure has a relative standard deviation of less than 5%.

Introduction

A procedure for the rapid assay of the proteolytic activity of stock bate using hide powder azure (HPA) as substrate was recently described.[1] (Azocoll could be used instead of HPA, but the latter is preferred since it does not require any pre-treatment for use, whereas the former does.[1]) Attempts to apply this assay procedure to samples of drum liquor (DL) taken during bating operations were unsuccessful. However, major revision of the procedure has rendered it suitable for measuring the proteolytic activity of DL. The development of the DL assay and examples of its application are described.

Materials

The substrate, hide powder azure (HPA), was purchased from Sigma Chemical Co., St. Louis, MO, or from Calbiochem, San Diego, CA. To ensure reasonably uniform, small particle size it was ground under liquid nitrogen in a Model 6700 Freezer/Mill from Spex Industries, Edison, NJ. Tris(hydroxymethyl)aminomethane (Tris) was the electrophoresis purity reagent supplied by BioRad Laboratories, Richmond, CA. Trichloroacetic acid (TCA) of analytical grade was supplied by Mallinckrodt, Inc., Paris, KY.

Buffer A was 50 mM Tris (Cl), 1.00 mM $CaCl_2$, pH 7.80 ± 0.05 (25°C). Incubation of drum liquor (DL) or diluted bate with substrate was carried out in a large Vacutainer brand tube

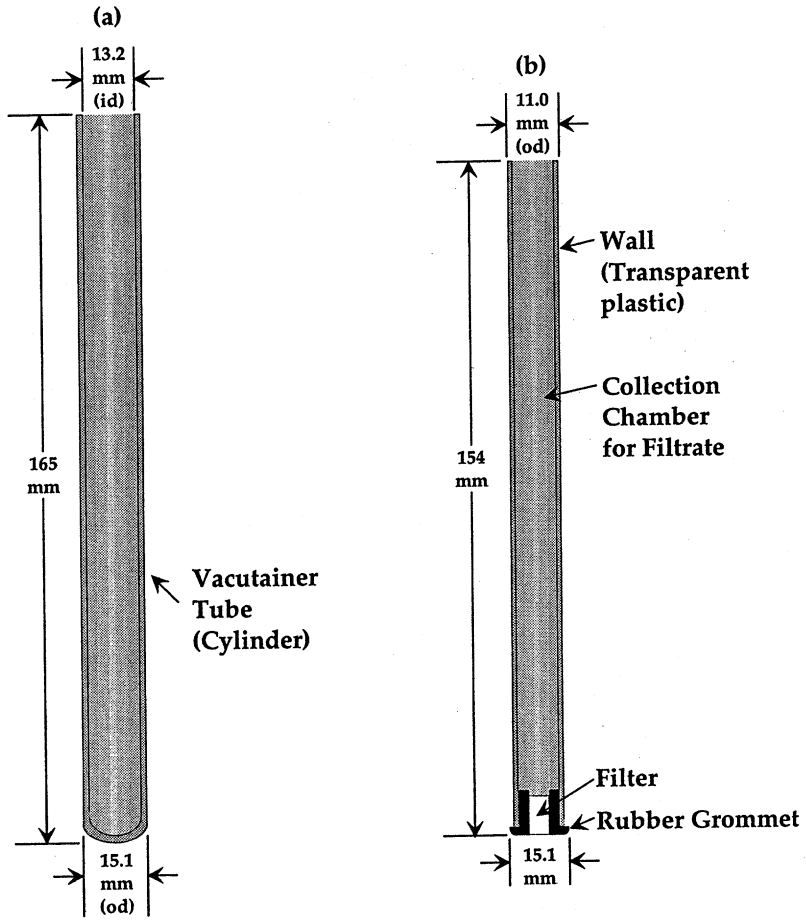


FIG. 1. — Longitudinal cross sections of (a) the Vacutainer tube ("cylinder") in which incubation and initial filtration were carried out, and (b) the serum filter ("piston") unit used for initial filtration. The dimensions given on the drawings are actual (measured) dimensions. The nominal dimensions used by the manufacturers are, for (a), 165 (ℓ) x 16 mm (od), and for (b), 16 mm (od) x 6" (ℓ). When the "piston" is inserted into the "cylinder", the outer, circular edge of the grommet is forced upwards, and forms a tight seal against the wall of the "cylinder."

(#6433) of the type used for blood collection (Fig. 1a), manufactured by Becton Dickinson Vacutainer Systems, Rutherford, NJ. The dimensions of the Vacutainer tube are 165 mm (length) x 15.1 mm (outer diameter); its nominal dimensions are 165 x 16 mm. Prior to mixing the tube was stoppered with a small Labcraft brand standard serum filter (#061-564) manufactured by Porex Medical, Fairburn, GA. The nominal dimensions of the small serum filter are 16 mm (od) x 4" (ℓ). It differs from the large serum filter (Fig. 1b) only in its length, which is 4" instead of 6". Agitation of the contents of the tube during incubation and again after addition of TCA was effected with a Roto-Torque heavy duty rotator, Model 7636, Cole-Parmer Instrument Co., Chicago, IL. Initial filtration of the tube contents after mixing with TCA was effected (after allowing time for large particles to settle) with a large Labcraft brand standard serum filter (#061-572, Fig. 1b). The dimensions of the large serum filter are 11.0 mm (od) x

154 mm (ℓ). The rubber grommet has an outer diameter of 15.1 mm. The nominal dimensions of the large serum filter are 16 mm x 6". When the serum filter (the "piston") is inserted into the Vacutainer tube (the "cylinder"), the periphery of the grommet bend upward and form a seal against the inner wall of the cylinder. The contents of the serum filter were filtered through a Millex AP 20 Prefilter and a 0.22 μm Millex-GV filter connected in series; these filters are manufactured by Millipore Corp., Bedford, MA. Absorbance measurements and spectra were obtained with a Lambda 7 UV/visible spectrophotometer manufactured by the Perkin-Elmer Corp., Norwalk, CT.

Results and Discussion

Modification of the Stock-Bate Assay

In the rapid assay of stock bate previously described,[1] bacterial bate, which was supplied in the form of a liquid suspension, was used directly. Pancreatic bate, which was supplied in solid form, was made up to a concentration of 40 g/l, to make the proteolytic activities (concentrations) of the two bate suspensions approximately equal. In either case, a 0.20 ml aliquot of the stock base suspension was diluted with 20.8 ml buffer and incubated with 100 mg substrate.

Since the concentration of bate in drum liquor (DL) is lower than that in the stock bate suspension by a factor of 20-50, it appeared that it might be possible to apply the stock bate assay to DL by taking a 5-10 ml aliquot of DL (in place of the 0.20 ml aliquot of stock bate), adding buffer to a total volume (DL + buffer) of 21 ml, and incubating with 100 mg substrate as before. Termination of the enzymatic reaction (by TCA), clarification (by centrifugation), measurement of solubilized dye, and calculation of activity would then be done in the same manner as for the assay of stock bate. However, when this was tried, the supernatant obtained on centrifugation was very turbid , making it impossible to evaluate the amount of dye solubilized by enzymatic action. On the assumption that the turbidity was due to the presence in DL of a relatively high concentration of Ca^{2+}, the solution was made alkaline and EDTA (ethylenediamine tetraacetate) was added to chelate Ca^{2+}. There was some reduction of the turbidity, but it was not eliminated. Citrate and NH_3 were also ineffective.

At this point it became clear that a major revision of the assay procedure would be necessary before it could be applied to DL. To minimize the problem with particulates, a substantial reduction in the concentration of DL in the incubation mixture was obviously in order. This required a compensating increase in the duration of incubation. To clarify the measured solution, it was decided to filter it through a 0.22 μm (pore-size) filter; filters of larger pore size were found to be inadequate. To make the method suitable for routine use in a tannery, the centrifugation step was eliminated and replaced by preliminary filtration through a serum filter (see Materials). With some samples, this filtrate could not be passed directly through the 0.22 μm filter; filtration through an intermediate prefilter was necessary. However, this does not extend the time required for filtration, since the prefilter and the final filter can be connected in series. Clarification is thus achieved by a 2-step process: (1) filtration with a serum filter, (2) filtration through a unit consisting of a prefilter and a 0.22 μm filter in series.

The stock bate assay was modified further for the sake of ease of operation and to minimize cost, as follows. (1) Incubation is carried out in the Vacutainer tube later used for preliminary filtration. (2) The incubation volume was reduced from 21 to 10 ml. (3) Mixing during incubation is done with a Roto-Torque Rotator (see Materials) instead of an orbital shaker.[1] The Rotator was also found to be convenient for mixing after addition of TCA. (4) The concentration

of the TCA reagent was doubled, so that a smaller volume of the reagent is required, thus reducing dilution of the dye whose color is measured.

Recommended Procedure

The modifications described above resulted in the following procedure for the assay of DL or stock bate. Materials referred to are described in the Materials section.

(1) Preliminary. Fifty mg of ground hide powder azure (HPA) are weighed into a large Vacutainer tube (the "cylinder", Fig. 1a). A large serum filter unit (the "piston", Fig. 1b) containing ca. 5 ml dry silica gel is inserted into the tube, leaving ca. 1 inch of the thin plastic wall of the piston extending above the top of the tube. The top of the piston is closed with a rubber stopper, and the assembly is stored, preferably at 5°C, until a few hours before the assay is started.

Samples of bate are prepared for assay as follows. For the assay of stock pancreatic bate, the solid is suspended in Buffer A to a concentration of 5.0 g/l. A liquid suspension of bacterial bate is diluted with 7 volumes of Buffer A. Drum liquor samples are not diluted prior to assay.

(2) Incubation. The stopper is removed from the piston and the piston from the tube. Nine ml Buffer A at room temperature are added to the tube (which contains the dry HPA). A 1.00 ml aliquot of undiluted DL or diluted stock bate is added to the Vacutainer tube (this defines time t = 0) and a small serum filter unit (piston) is inserted into the tube until the bottom of the piston is ⅛-¼ inch from the liquid surface. The tube-and-piston assembly are mounted on the circumference of the Rotator at an angle of 45° to the axis of the rotation, which is horizontal, and the Rotator is operated at 100 rpm (the maximum speed obtainable). At time t = 19 min, the tube is removed from the Rotator and the piston from the tube. At t = 20, 5.0 ml TCA are added, the piston is reinserted, and the contents of the tube are mixed on the Rotator for 2-5 min.

(3) Removal of solids. After it is removed from the Rotator the tube is allowed to stand for 2-10 min undisturbed, to allow large particles to settle. The small piston is removed and replaced with a fresh, large piston (serum filter). The latter is depressed until it is close to the blue precipitate at the bottom of the tube; compression of the precipitate is avoided. The contents of the serum filter are withdrawn into a 20 ml syringe and filtered through a Prefilter and a 0.22 μm filter, connected in series.

(4) Colorimetry. The absorbance (A) of the filtrate is measured at 594 nm and, if an objective evaluation of possible turbidity is desired, at 445 nm. If $A_{594}/A_{445} = 13$, there is no turbidity, and A_{594} provides a measure of the dye concentration. The spectrum of a typical clear filtrate is shown in Fig. 2.

Standard Curve

A typical curve expressing the dependence of absorbance on bate concentration (C_E) is shown in Fig. 3. The curve varies, of course, with the particular bate used and with the temperature of incubation. For the graph shown, the bate was Rohapon 6000 and the temperature was 22°C. Since the mixer used does not provide for controlling the temperature of the samples during incubation, it is important to run the unknowns and standards at the same time, or at least to ensure that they are run at the same environmental temperature.

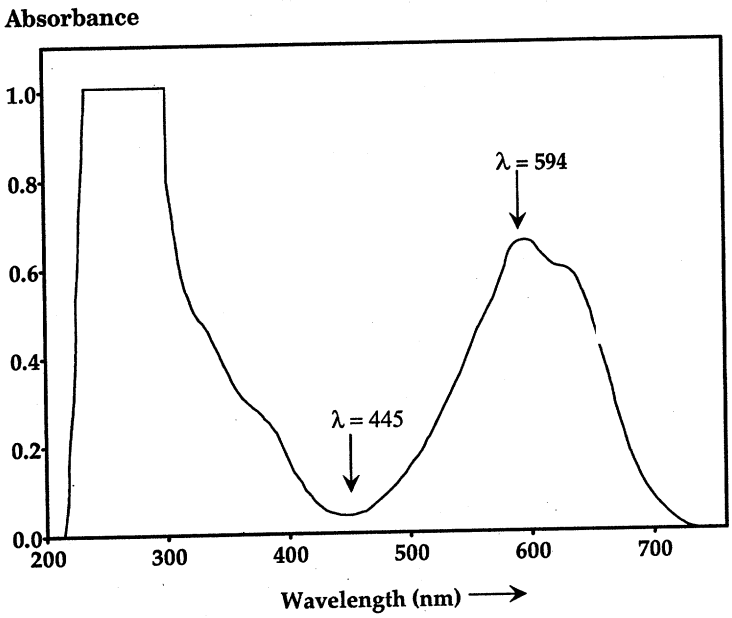


FIG. 2. — Spectrum of a typical clear filtrate. The absorbance of solubilized blue dye at 594 nm provides a measure of the extent of enzymatic hydrolysis. Turbidity in the filtrate results in an increase in the absorbance at 445 nm relative to that at 594 nm.

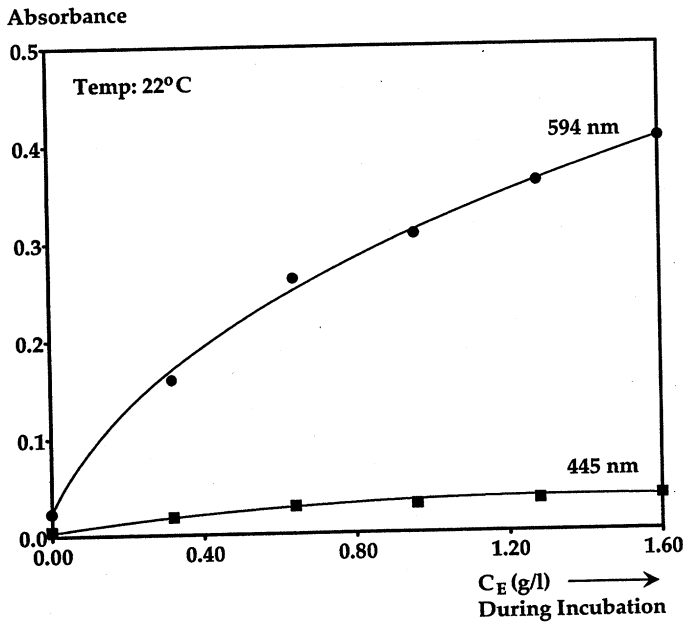


FIG. 3. — Calibration curve for determining the concentration of proteolytic activity (C_E) in a sample of DL or stock bate. C_E is expressed in terms of the weight of solid pancreatic bate (Rohapon 6000) per unit volume. The incubation temperature was 22°C.

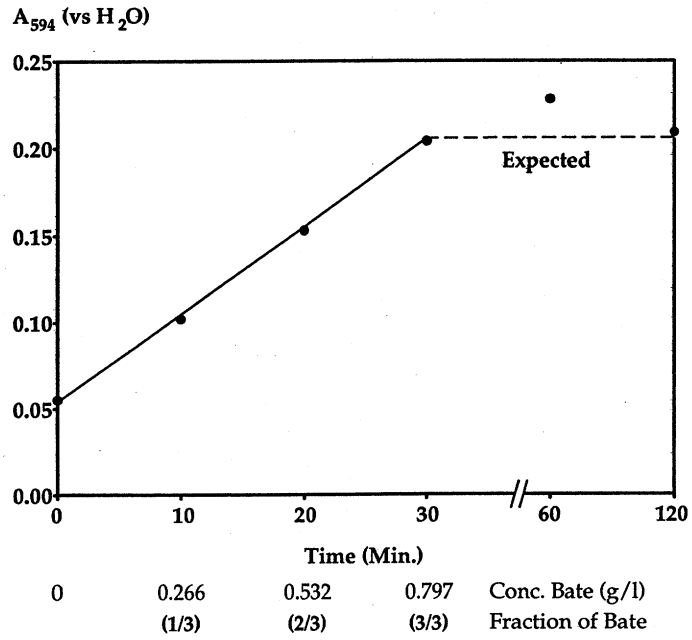


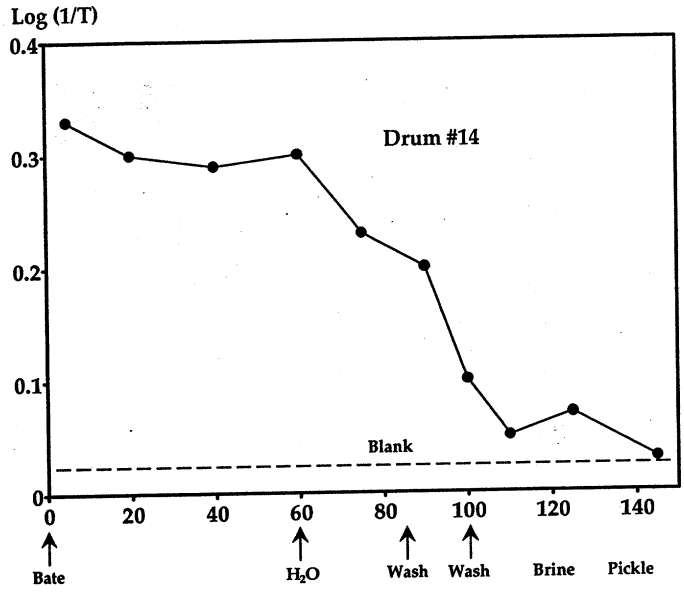
FIG. 4. — Proteolytic enzyme concentration, as measured by the recommended assay procedure, in DL before and after addition of bate to the drum. The bate was added in 3 equal portions at times t = 5, 15, and 25 min, and samples were taken for assay at t = 0, 10, 20, 30, 60, and 120 min.

Application of the Assay Procedure to Drum Liquor

The assay procedure described above was tested during a bating operation in a pilot-plant drum (Fig. 4). One third of the bate was added at time t = 5 min, and equal amounts were added at t = 15 and t = 30 min. The final bate concentration was close to 0.8 g/l. The DL was assayed for proteolytic activity at t = 0, 10, 20, 30, 60, and 120 min. As shown, the measured absorbance accurately reflects the concentration of added enzyme in the DL.

The assay procedure was also applied to DL samples taken from two commercial drums during and after bating (Figs. 5a and 5b). After the addition of bate (at time t = 0) the concentration of proteolytic enzyme in the DL was essentially constant until dilution and washing. After the second wash the enzyme level fell to very low levels, as expected.

The proteolytic enzyme level was then measured in 9 commercial drum-loads in normal production 20 min after the addition of bate (Fig. 6). The relative standard deviation of the enzyme levels is 12%. However, DL contains at least one substance, viz., Ca^{2+}, which activates/protects some proteolytic enzymes and probably contains some enzyme inhibitors. Lack of control by the tanner of the concentrations of these modifiers of enzyme activity from drum to drum, together with the results shown in Fig. 6, suggest that it may be worthwhile to assay DL early in the bating operation so that the level of proteolytic activity might be adjusted to the level desired. Such control of the activity level could be critical when evaluating the effect of different enzyme levels on the quality of the product.



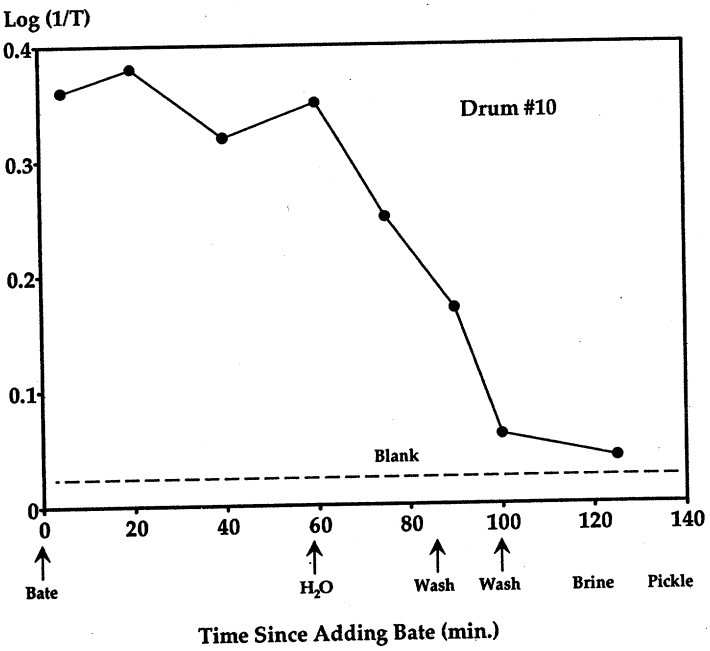


FIG. 5 (a & b). — Proteolytic enzyme concentration, as measured by the recommended assay procedure, in two commercial drums during and after bating of hides. The bate was added at time t = 0.

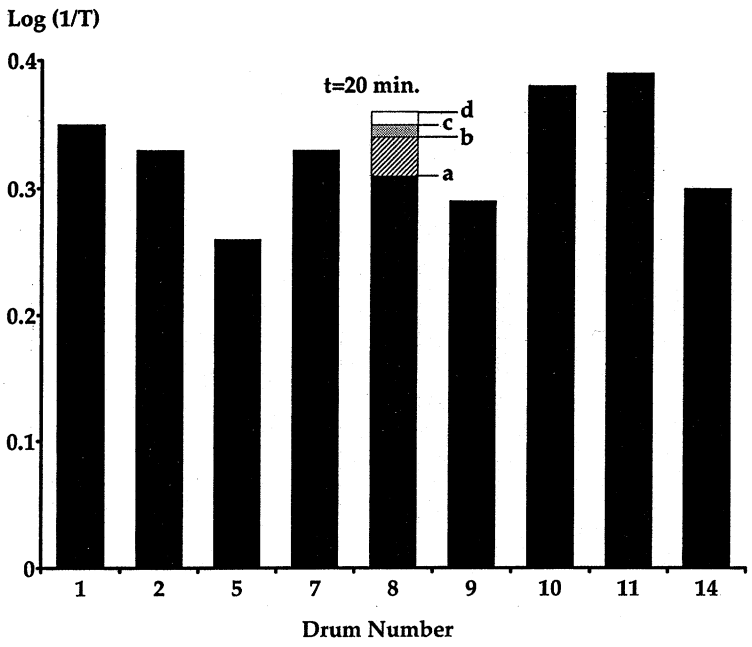


FIG. 6. — Proteolytic enzyme concentration, as measured by the recommended assay procedure, in 9 drums 20 min after addition of bate. Four assays were done on the DL of drum #8, as indicated (a-d). A single assay was done on the contents of each of the other drums.

Addendum

After completing the work described above, it was found that with some hide powder azure, (HPA) the dependence of absorbance on enzyme concentration plateaus below an enzyme concentration of 0.64 g/l (cf. Fig. 3). This indicates that, for such HPA, the ratio substrate/enzyme is inadequate. This problem can be circumvented by reducing the enzyme concentration, reducing the incubation time, or increasing the amount of substrate. The problem will be dealt with quantitatively in a forthcoming communication.